REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
xx-11-2011	Presentation	Oct 2011 - Nov 2011

4. TITLE AND SUBTITLE
Wide Area Recovery and Resiliency Program (WARRP)
Presentation - Feasibility of Wide-Area Decontamination of Bacillus anthracis Spores Using a Germination-Lysis Approach

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

5. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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8. PERFORMING ORGANIZATION REPORT NUMBER
LLNL-PRES-508394

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Lori Miller
Department of Homeland Security
Science and Technology Directorate
Washington, DC 20538

10. SPONSOR/MONITOR'S ACRONYM(S)
DHS

11. SPONSOR/MONITOR'S REPORT NUMBER(S)
3.2.1

12. DISTRIBUTION / AVAILABILITY STATEMENT
Dist A:

13. SUPPLEMENTARY NOTES
 The WARRP Program is a joint effort of DOE, DoD, EPA, HHS, and DHS.

14. ABSTRACT
This presentation covered background and problem, spore resistance to decontamination, current solutions, the Germination-Lysis approach, proof of concept, germination rate, relative costs, potential for scale-up, and challenges and possible solutions.

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15. SUBJECT TERMS
WARRP, Bacillus anthracis, Anthrax, Germination-Lysis, Wide-area Decontamination

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Peggy West
U	U	U	UU	21	19b. TELEPHONE NUMBER *(include area code)* (619) 553-6899

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Feasibility of Wide-Area Decontamination of Bacillus Anthracis Spores Using a Germination-Lysis Approach

2011 CBD S&T Conference

November 16, 2011

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LLNL-PRES-508394

This work was performed under the auspices of the U.S. Department of Energy by Lawrence Livermore National Laboratory under contract DE-AC52-07NA27344. Lawrence Livermore National Security, LLC

Talk Outline

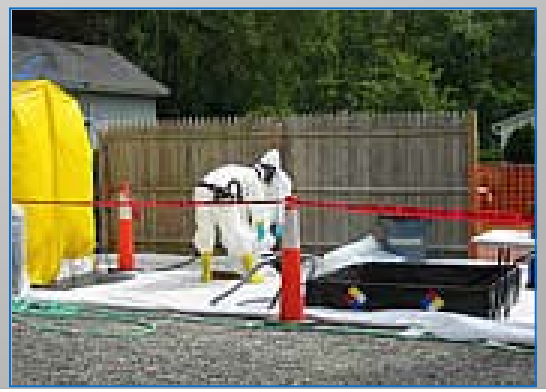
- Background & Problem: Spore Resistance to Decontamination
- Current Solutions
- Germination-Lysis Approach
- Proof of Concept
- Germination Rate –Timing
- Relative Cost
- Potential for Scale-up
- Challenges and Possible Solutions

Few Options Exist for Wide-Area Outdoor Decontamination of B. anthracis Spores

- **Gruinard Island**
  5% formaldehyde

- **Sverdlosk Release**
  UNKNOWN: but washing, chloramines, soil disposal believed to have been used

- **Danbury, Connecticut**
  nonporous surfaces treated with ~0.5-0.6% pH-amended bleach



Sources:
Manchee et a l., 1994, Meselson et al., 1994 , EPA, 2007
http://petra.wijnsema.nl/pictures/train_trip.htm
http://yosemite.epa.gov/opa/admpress.nsf/names/r01_2007-12-12_danbury
http://www.thesahara.info/medical/anthrax_gruinard_island.jpg
http://news.bbc.co.uk/olmedia/1455000/images/_1457035_gruinard_island_150map.gif

EPA Testing on Outdoor Materials Provide Options for Hard Nonporous Surfaces

Disinfectant	>6 Log Reduction on Materials (EPA, 2010a,b; Wood et al., 2011)	Target Disinfectant Concentration (ppm)	OSHA PEL (ppm)	CDC IDLH (ppm)
pH-amended bleach (sodium hypochlorite)	Stainless Steel, Glass, Aluminum, Porcelain, Granite, Concrete, Brick, Butyl Rubber	6000	1	30
Hydrogen peroxide /peroxyacetic acid (Peridox, Spor-Klenz, Oxonia)	Stainless Steel, Glass, Aluminum, Porcelain, Granite, Brick, Treated Wood, Butyl Rubber, Galvanized Metal	Various formulations	10	75
Aqueous chlorine dioxide (ClO_2) (DioxiGard, SanDes)	Galvanized Metal, Glass	500	0.1	5
Hydrogen peroxide and other agents (Decon Green)	Stainless Steel, Glass, Aluminum, Porcelain, Granite, Brick, Butyl Rubber	35,000	1	75
Sodium dichloroisocyanurate (CASCAD)	Stainless Steel, Glass, Aluminum, Porcelain, Granite, Concrete, Brick, Asphalt, Treated Wood, Butyl Rubber	Per formulation	NA	NA

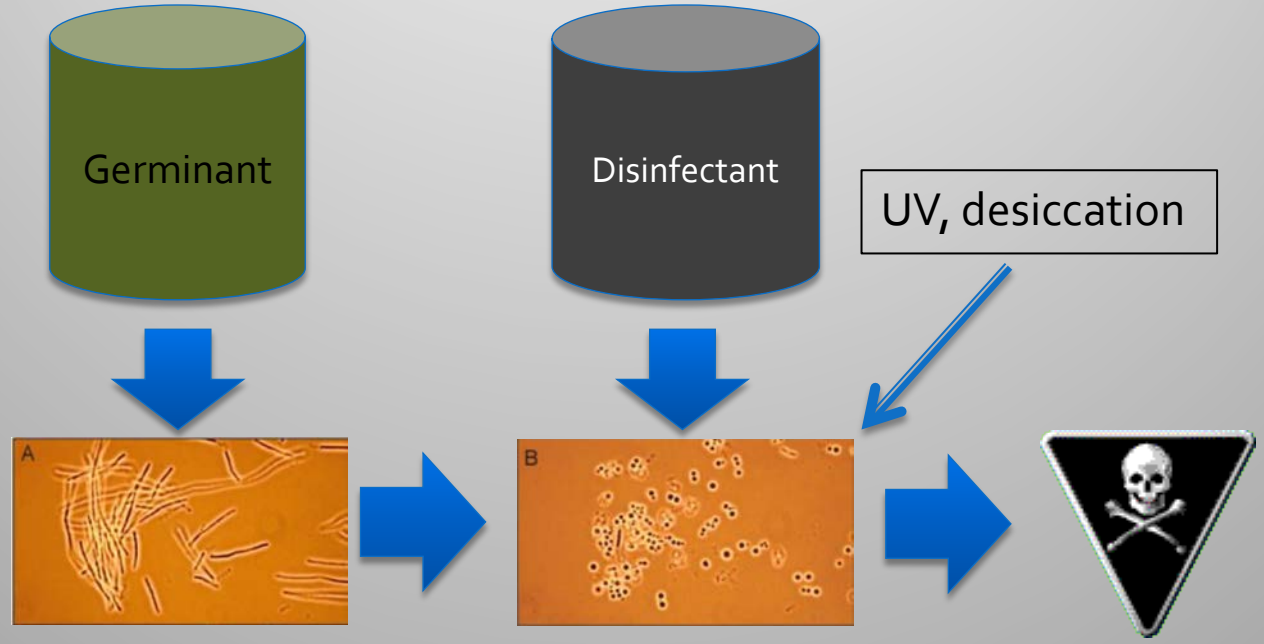
Challenges for Outdoor Decontamination of *B. anthracis* Spores

- High disinfectant concentrations increase operational costs and risk to human health during application
- Disinfectants are corrosive, damaging to surfaces/materials
- Disinfectants may be consumed in organic/chemical backgrounds in the environment
- Decontamination activities could promote spore transport in liquid or air phase (reaerosolization), or via fomites
- No disinfectants have been demonstrated to be effective in soils or on vegetation
- Disinfectants pose long-term human health and environmental impacts

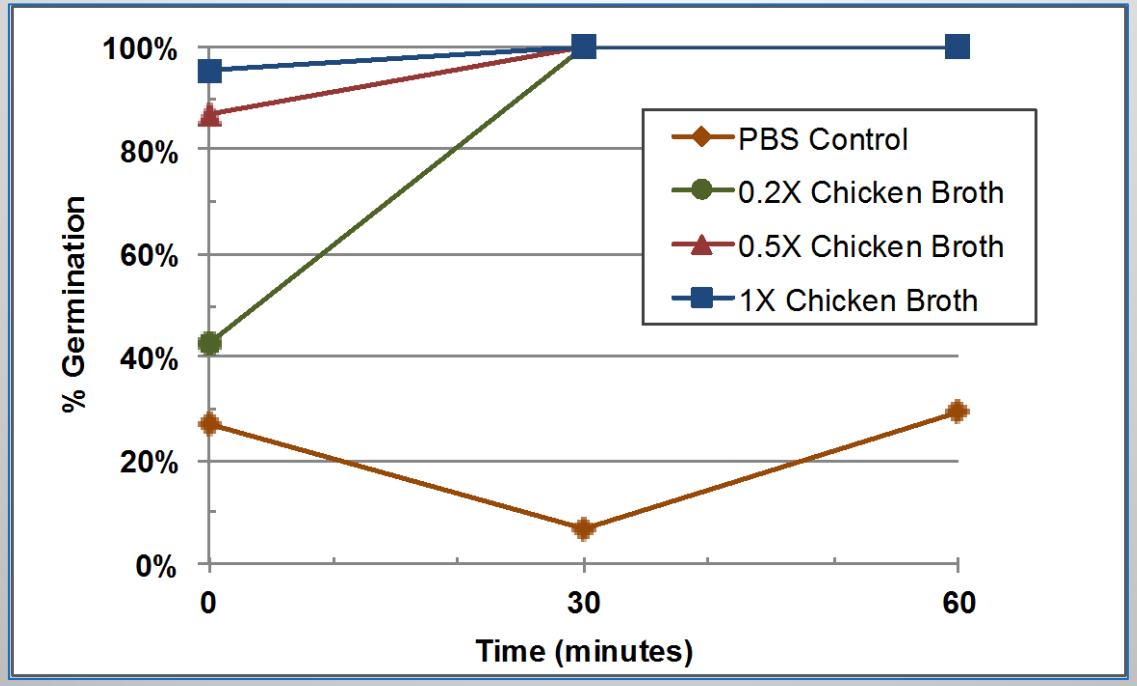
The Germination-Lysis Approach

At acceptable environmental conditions and on appropriate surfaces, spores are germinated and then disinfected. Germinated spores and vegetative cells are more susceptible to simple disinfection methods, including UV exposure and desiccation.

- **Option 1**: Apply germinant and wait 15 to 30 min and then apply disinfectant solution (or demonstrate natural attenuation).
- **Option 2**: Apply germinant with a time released disinfectant solution (*if feasibility is demonstrated*).



Proof of Concept: Low cost, readily available materials may be effective



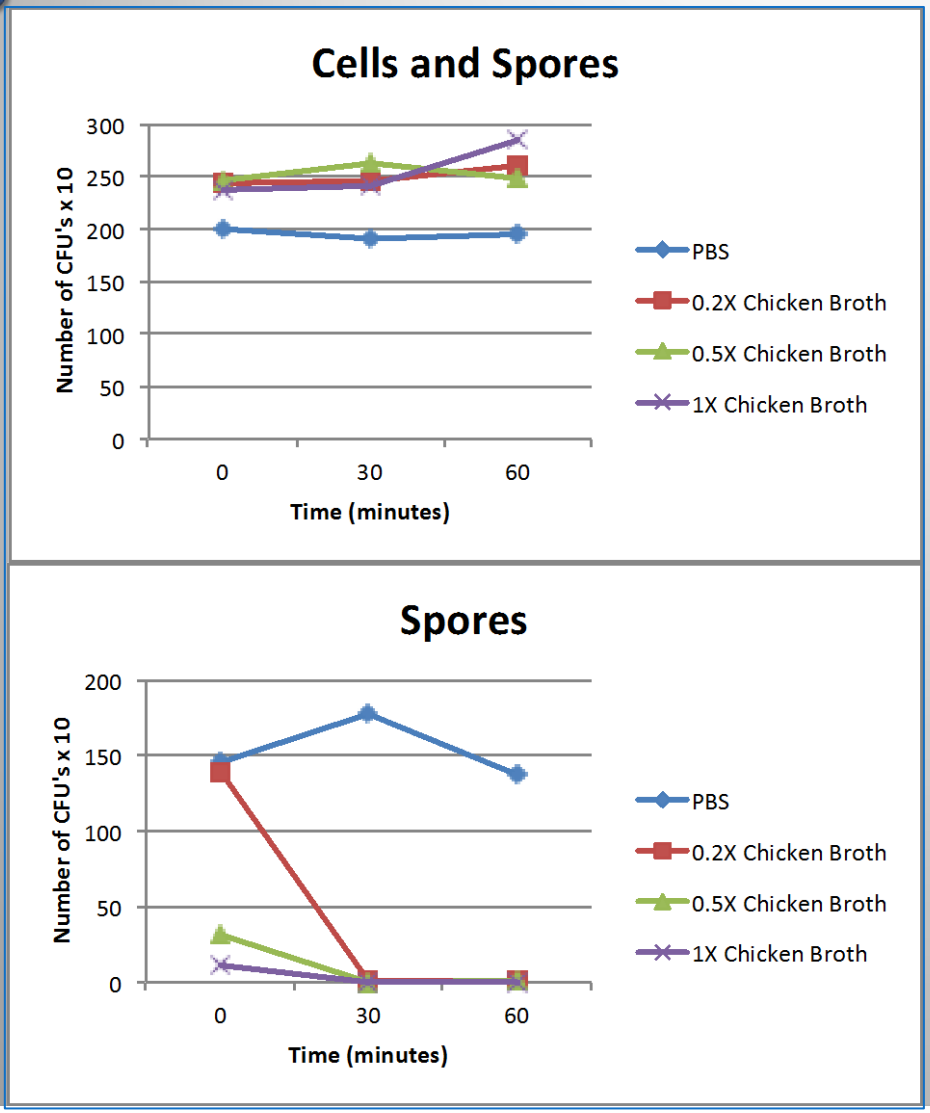
- Percent spore germination determined by heat treatment relative to the total population (cells and spores) from plate culture analysis (starting with ~10^4 spores/mL).
- Filtered, fat-free chicken broth was diluted in phosphate-buffered saline.
- Time o represents 10-15 min exposure before analysis could be conducted.

Germination kinetics relative to decontamination kinetics

- Chicken Broth – 15 to 30 min germination
- L-Alanine/inosine – 15 to 30 min germination
- Casamino acids/inosine and Proteose Peptone/inosine – 30 min germination
- Typical decontamination contact times vary between 30 to 120 min (pH amended bleach, Hydrogen peroxide /peroxyacetic acid, etc..)

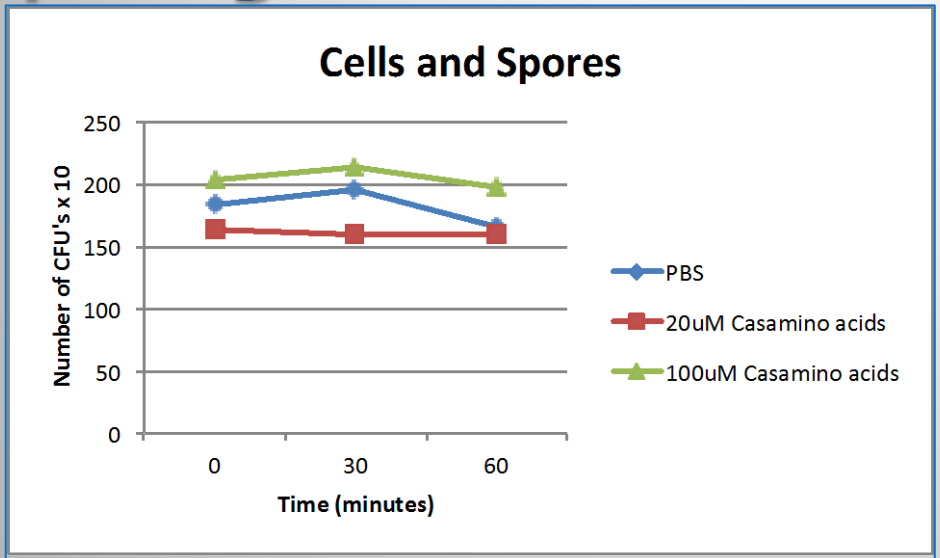
Our preliminary data in solution indicate that germination can occur on time scales relevant for wide-area decontamination activities

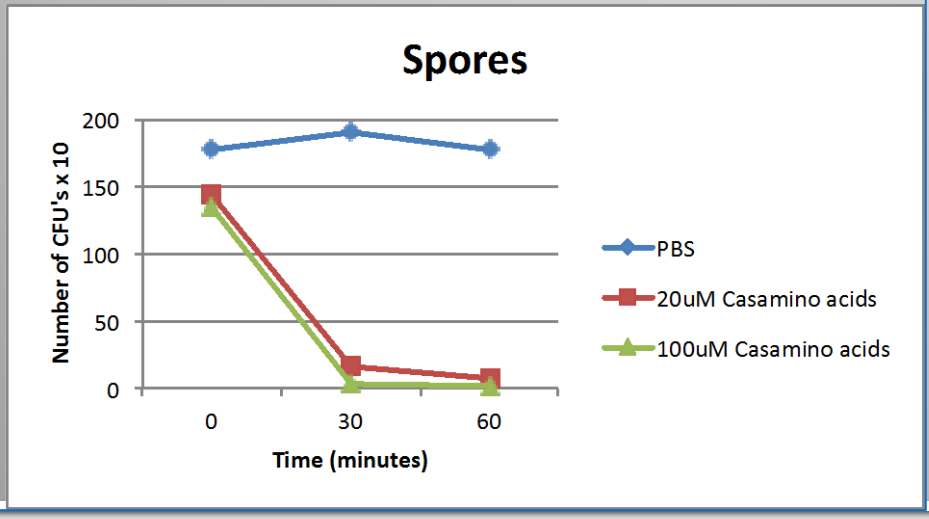
B. anthracis Sterne spores showed rapid germination with dilute chicken broth



- Rapid germination immediately after broth addition (relative to control, phosphate-buffered saline, PBS)
  - 1X – 93% germination
  - 0.5X – 78% germination
- 100% germination at 30 min with 0.2X, 0.5X and 1X broth
- Average of 3 replicates shown
- Determined by direct plating for cells and spores
- Determined by heat treatment (65°C for 20 min) for spores
- Broth was filter-sterilized and pH adjusted (pH = 7.4)

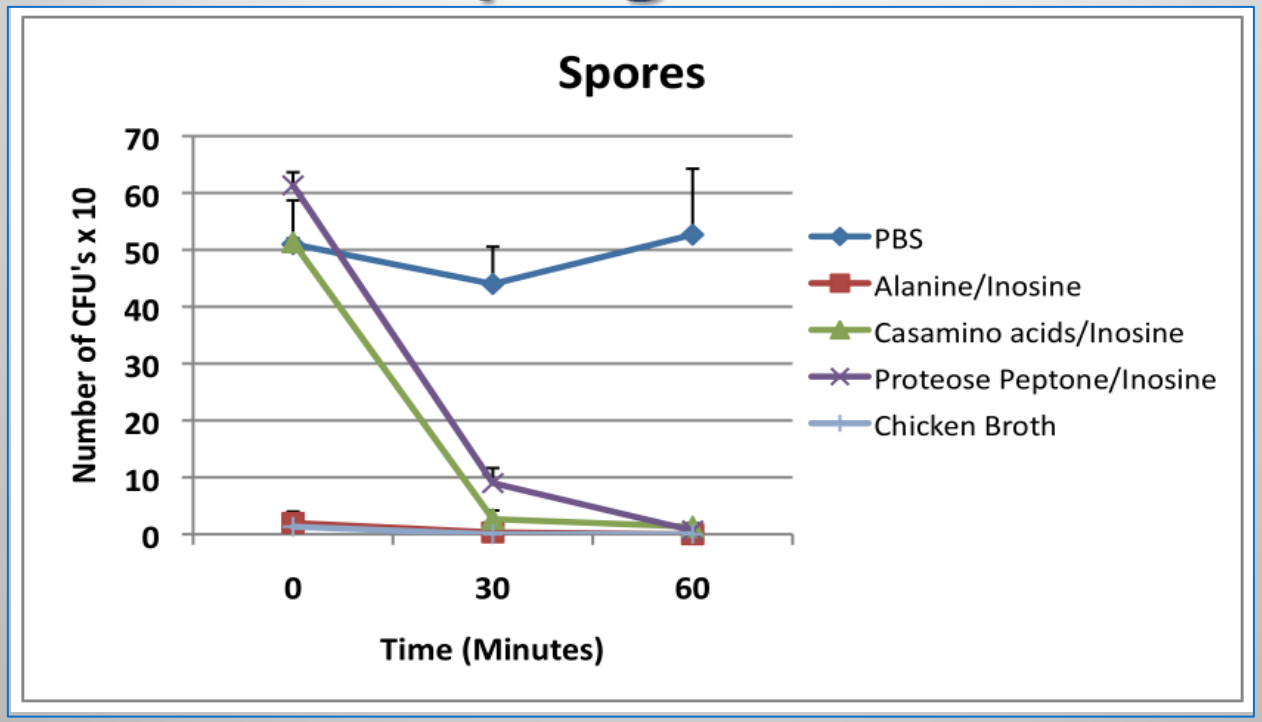
Dilute media components showed rapid spore germination





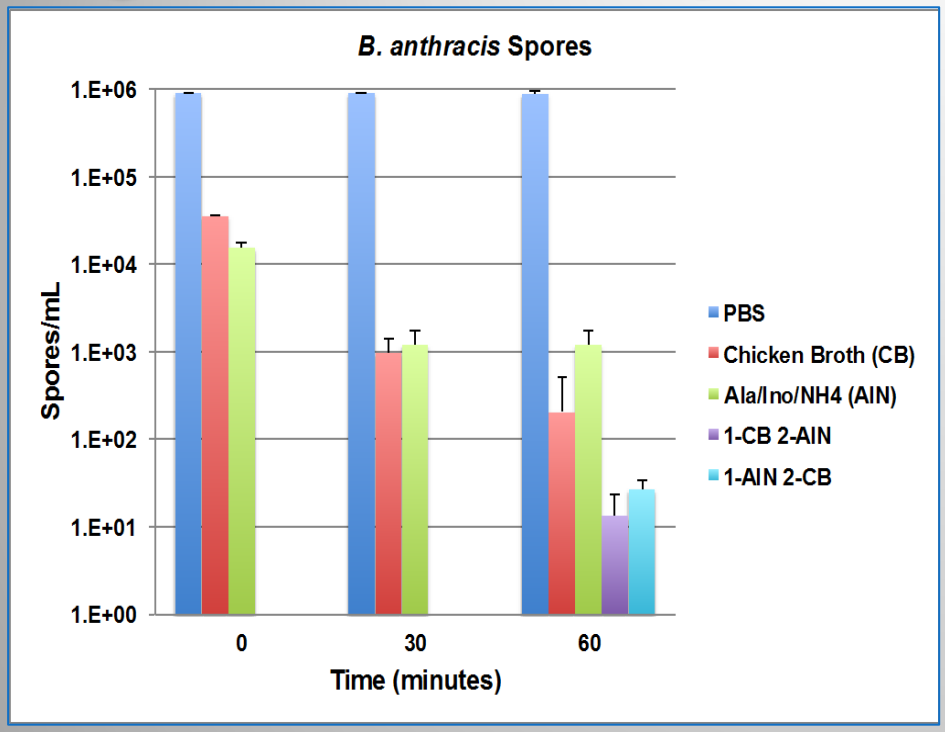
- Nearly 100% germination at 30 min with 20 µM casamino acids/100 µM inosine
- Determined by direct plating for cells and spores
- Determined by heat treatment (65°C for 20 min) for spores

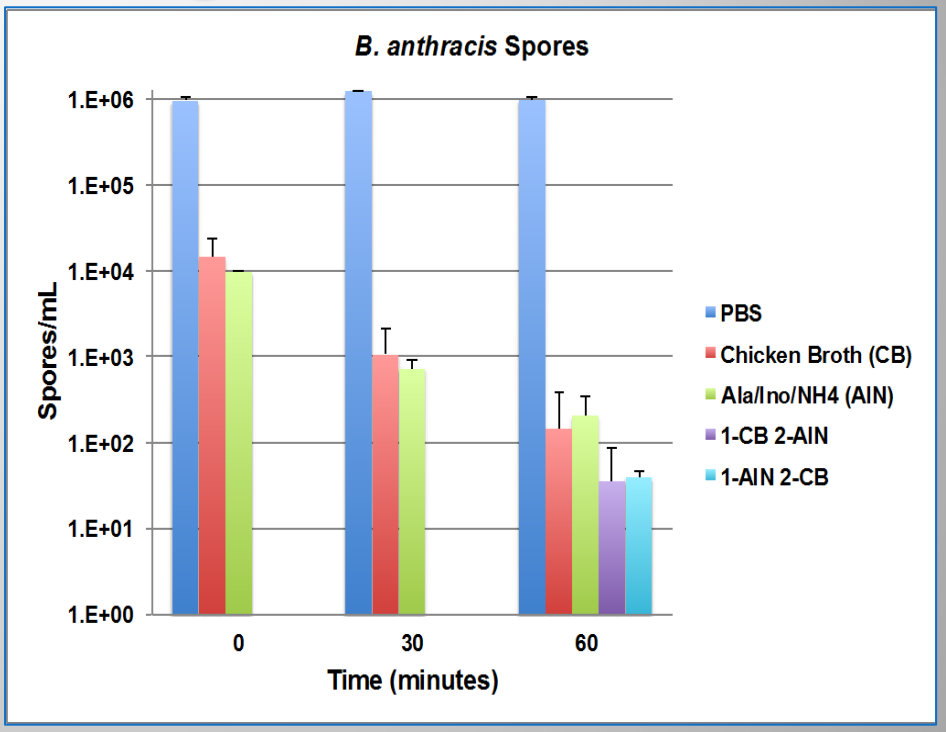
Summary: *B. anthracis* Sterne spores treated with simple germinant solutions



- Chicken Broth and L-alanine/inosine effective in first 15 min (time to process samples) for low spore levels (10^2-10^3)
- Casamino acids/inosine and Proteose Peptone/inosine effective in ~30 min
  - Casamino acids: 2% free Alanine; Proteose Peptone: 0.5% free Alanine; Inosine concentration not known

Subsequent addition of another germinant may enhance germination

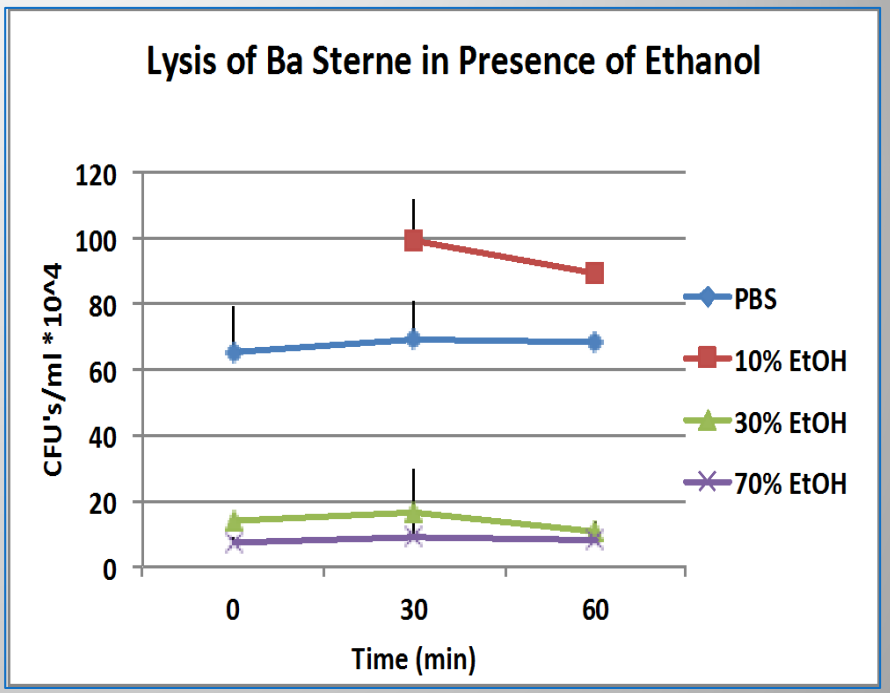




- 10^6 *B. anthracis* Sterne spores; 1X filtered, fat-free chicken broth (CB)
- Addition of alanine/inosine/NH_4Cl (AIN) to spores in CB or addition of CB to spores in AIN showed higher percent germination
- Need to compare with addition of same germinant
- Results for 30°C and 25°C were consistent; > 4 log germination

B. anthracis cells treated with lysis materials

- 30% and 70% ethanol showed disinfection within 15 min
- **5% pH-adjusted bleach was effective within 15 min (100% lysis)**
- **3% H_2O_2 was effective after 30 min (100% lysis)**
- Salt up to 30% was only partially effective at cell lysis
- 5% acetic acid (vinegar) was ineffective
- Analysis of natural degradation (UV exposure, desiccation) is ongoing



Challenges to Germination-Lysis Decon

- Levels of germinants and disinfectants needed
- Materials may be consumed in organic/chemical backgrounds in the environment
- Maintenance of optimal environmental conditions for activity
- Kinetics of processes may be too slow relative to maintaining optimal conditions
- Impact of nutrient source on indigenous populations (fouling)
- Spore transport (liquid or air phase)
- Superdormancy or incomplete germination (challenge for higher spore densities)
- Incomplete lysis—propagation of pathogen cells/spores
- Need to confirm that surrogate (Sterne) responds like virulent *B. anthracis*

Relative Cost

- Chicken broth estimated material cost = $5 to $11 per gallon germination solution (0.5x), ($2 to $5 at 0.2x solution)
- Casamino acids estimated material cost = $0.06 per gallon germination solution
- Alanine/Inosine estimated material cost = $0.05 per gallon germination solution
- Clorox bleach estimated material cost = $0.4 to $0.9 per gallon decontaminant solution

Potential Required Decontaminant Volumes

Conservative estimates for a 30-min contact time:

- Impervious surfaces (e.g., glass or plastic) = 1,400 gal/ac.
- Concrete = 3,500 gal/ac (assuming 10% porosity) and 0.25-in material penetration depth.
- Asphalt = 4,200 gal/ac (assuming 20% porosity) and 0.25-in. material penetration depth.
- Soils (NRCS Hydrologic Group C and NRCS Hydrologic Group A) = 4,800 to 83,600 gal/ac.

Potential for Scale-up

Type of application equipment	Volume capacity (gal)	Reported liquid application rate (gpm)	Apparatus cost[b] ($)
Plot scale—limited or personnel decontamination (<0.5 acre)[c]			
Backpack sprayers Portable sprayers (on dolly or rollers)	2 to 50	<10	≤10K
Mesoscale—small structures or roads (0.5 to 5 acres)			
Skid-mounted spray systems Horizontal boom sprayers Tree sprayers Vertical boom sprayers	100 to 3,200 (modular)	<100	10K to ≥100K
Large-scale—large buildings, ports, or parking lots (5 to 50 acres)			
Fire trucks, fire boats, and hydrants Agricultural sprinklers Small aircraft	120 to 2,000	1,000 to 1,500 trucks and hydrants up to 20,000 fire boats (total) 74 to 695 sprinkler heads (aircraft not available)	10K to 100K
Wide-area—large, uniform areas (not applicable to high-permeability soils) (>50 acres)			
Larger aircraft (C-130 or DC-10) Super tanker aircraft (747)	3,000 to 20,000	Not available	50K to ≥100K

[a] Data from DHS (2007); Cal Fire (2010); Hsu (2006); company websites; and customer sales representatives.
[b] Approximate cost for equipment purchase or rental does not include staffing, reagents, or other deployment costs.
[c] Area scales estimated for ~1 day (4 hr for application, 2 hr for setup, and 2 hr for teardown). When estimating acreages, include total vertical and horizontal surfaces to be treated.

Agricultural and Other Spray Technologies

- Liquid application rates: 10^{-3} to 1 L/m^2
- Deposition layers: 1 to 10^3 µm
- Work rates: 3 to 600 ha/hr
- Droplet size (diameter): 10 to 10^3 µm
- Droplet velocities: 10^{-1} to 10^1 m/s


http://www.kirtland.af.mil/photos/mediagallery.asp?galleryID=2467


http://www.spraycontractor.com/


http://www.aero-news.net/index.cfm?do=main.textpost&id=07a7bbca-6124-4eae-ab7f-94f9806c9cc0

Opportunities & Possible Solutions

- Synergistic reactions to multiple germinants (e.g., Yi et al., 2011 J. Bacteriol. 193:4664)
- Novel lytic proteins for *B. anthracis*—1) germination specific lytic enzymes and 2) endolysins for vegetative cells
  (Dr. Paul Jackson & collaborators at LLNL)
- Possible combined germinant and lytic agent applications
  (Dr. Paul Jackson & collaborators at LLNL)
- Advanced agricultural spray technologies (Dr. Ken Giles, UC Davis)
- Work with collaborators in the LLNL Select Agent Facility for testing with virulent *B. anthracis* to confirm surrogate results
- Increased understanding of spore germination will enhance our ability to optimize the germination-lysis process

Collaboration

- **LLNL colleagues:**
  - Dr. Paul Jackson, Dr. Matt Coleman (enzyme-based decon approaches, experimental systems for testing)
  - Dr. Joe Tringe (engineering and spray technology for other applications)
- **Partner with UC-Davis colleagues for dissemination technology**
  - Dr. Ken Giles
    Optimization of germinant and disinfectant application (formulation and delivery parameters)
- **Partner with US EPA National Homeland Security Research Center (NHSRC):**
  - Dr. Worth Calfee, Dr. Shawn Ryan
    Experimental design and data analysis of microcosm studies and EPA chamber studies

Acknowledgments

- Feliza Bourguet, Jessica Wollard, Gloria Murphy, Teneile Alfaro (LLNL)
- DHS Wide Area Recovery and Resiliency Program (WARRP) and Interagency Biological Restoration Demonstration (IBRD) - Chris Russell & Lance Brooks